The Reproductive Toxicity of Boric Acid

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Previous studies on the reproductive toxicity of boric acid have indicated that male rodents suffer testicular atrophy after treatment. There were, however, no studies of the potential effects on female fertility or on the neonate. In addition, no study described the development of the testicular lesion, thought to be related to the mechanism of toxicity. A Reproductive Assessment by Continuous Breeding (RACB) study using mice exposed to boric acid at 1000, 4500, and 9000 ppm in the diet indicated that there are probably multiple sites of action, although male fertility appears very sensitive. Possible effects on female fertility cannot be separated from potential developmental toxicity and need additional investigation. Decrements in sperm motility were observed at all exposure levels, and testicular atrophy was confirmed in high- and middle-dose-group males. This was investigated further by timed serial-sacrifice studies using 9000 ppm in the diet of rats, which found that the first lesion seen in the testis was an inhibition of spermiation (release of mature spermatids). With continued dosing, this was followed by a disorganization of the normal ordered layering of the seminiferous epithelium, germ cell sloughing and death, and finally, atrophy. Subsequent studies using additional doses (2000, 3000, 4500, 6000, and 9000 ppm) found that it was possible to observe inhibited spermiation that did not progress to atrophy (4500 ppm and below) within the 9-week exposure period. Also, once atrophic (from 9000- and 6000-ppm exposures), there was no return of spermatogenesis after refeeding of normal diet for 8, 16, 24, or 32 weeks, despite the presence of a normal-sized population of spermatogonia that was seen to divide. No effects were observed in rats treated with 2000 ppm. Testicular boron concentrations remained steady during exposure at the same level as found in blood, and declined to background levels within 72 hr after cessation of exposure. Bone boron levels were greater than those found in blood, and maintained slightly elevated levels even 32 weeks after the cessation of exposure. These studies document the effects of boric acid on functional and structural aspects of the male reproductive system; demonstrate that the tissue concentration of boron, and not total dose, is important for the testicular toxicity; and show that there is a threshold for these effects. These studies also suggest that the potential for recovery from boric acid-induced testicular atrophy should be examined in other species to evaluate the significance of the persistent atrophy seen in the rats. — Environ Health Perspect 102(Suppl 7):87–91 (1994)

Key words: boric acid, spermatogenesis, rats, inhibited spermiation, atrophy, recovery from, threshold

Introduction

Boric acid has been identified as a reproductive toxicant for at least the past 20 years. The studies by Weir and Fisher in 1972 (*1*) established boric acid as toxic to the male reproductive system, the skin, and the CNS of the young rat, as well as an inhibitor of growth (*2*). This was followed in 1978 by studies by Lee and colleagues, who detailed the effects of borax on the testis (*3*). They found testicular degeneration and infertility after 60 days of boron consumption. Other studies have reported adverse effects of boron exposure on the male reproductive system (*4*). These studies raised the issue of the reproductive toxicity of high-level boron exposure. No study can be comprehensive; however, these studies left a need for an adequate evaluation of male fertility, as well as a correlation of testicular boron levels to lesion development. Female fertility was not addressed in these studies at all, nor was developmental toxicity.

The National Institute for Occupational Safety and Health asked its sister agency in the National Toxicology Program, National Institute of Environmental Health Sciences (NIEHS), for additional data to use in writing exposure guidelines for boric acid. Thus, we began a series of studies designed to evaluate functional aspects of reproductive toxicity. In the follow-up studies, we wanted to see how the testicular lesion developed, because the initial series of changes in the injured testis is thought to be related to the mechanism of toxicity. Initial observations on pathogenesis might shed some light on boron's mechanism of toxicity. We used multiple dose levels in an attempt to dissect the various parts of the lesion, to see if one led irreversibly to the next. We wanted to correlate all of these to circulating and testicular boron levels. As a side issue, we wanted to see if bone acted as a depot for boron, and thus might be both another target organ for boron toxicity and a complicating factor in the estimations of internal dosimetry.

Methods

RACB Study

Specific methods and results for the Reproductive Assessment by Continuous Breeding (RACB) study may be found in Fail et al. (*4*). Briefly, male and female Swiss CD-1 mice (11 weeks of age at start) were fed boric acid in a powdered diet starting 1 week prior to cohabitation. Boric acid levels were 1000, 4500, and 9000 ppm. Animals were cohabited for 14 weeks; pups were evaluated as the litters were born, and then humanely euthanatized. The cohabitation period was followed by a 6-week separation period, to allow for the delivery and rearing of the last litter. After weaning this litter, a test to determine the most-affected sex was performed by mating animals from the middle-dose group with controls. After the delivery of the resulting litters, the F_0 mice were euthanized and necropsied. The F_1 mice were reared to sexual maturity while consuming the same diet administered to their parents, and mated to nonsiblings within the same dose level. The resulting

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litter was evaluated, and the F_1 animals were euthanized and necropsied.

Male Pathogenesis Study

This study was performed to define the steps in the development of the testicular lesion (*5*). Several small pilot studies were performed to ascertain that boric acid would affect rats, and to estimate appropriate sacrifice times. For the definitive study, 6 treated and 4 control rats were deeply anesthetized and perfused with aldehyde fixative after 4, 7, 10, 14, 21, and 28 days of exposure to a diet containing 9000 ppm boric acid. Only a single dose level was used in this experiment. A hormone study was performed because the testicular lesion appeared to be similar to one caused by androgen insufficiency. This was evaluated on days 4, 7, 10, and 28.

Dose/Time Study

The pathogenesis study above raised several questions: what was the relationship between testis boron and lesion development? Was total boron exposure important, or did dose *rate* determine the nature of the testis lesion? Could the various characteristics of the lesion be separated using different doses? That is, would it be possible to develop the first part of the lesion without progressing to the second? If so, it might suggest that separate mechanisms were operating for these different aspects of the lesion; alternately, if a single mechanism were operative, it would identify the places where this process was physiologically important. And what was the role of bone in the internal disposition of boron?

In preliminary pilot studies, three rats/group/time consumed diet containing 2000, 3000, 4500, 6000, and 9000 ppm BORIC ACID, and were killed at weekly intervals for up to 9 weeks. In the definitive study, young adult male rats were exposed to boric acid-containing powdered feed at 0, 3000, 4500, 6000, and 9000 ppm, and 6 rats from each group were sacrificed at weekly intervals for 9 weeks (*6*). End points were body and organ weights, numerous sperm parameters, histology of selected organs, clinical chemistries, and boron content in blood, testis, and tibia/fibula.

This study also incorporated a recovery assessment. There were several questions being addressed: what were the kinetics of boron loss after cessation of exposure? Would spermatogenesis recover after atrophy, and how long would this require? To address these, some animals from the 0, 9000, 6000, and 4500 ppm groups were returned to pelleted control feed after the 9-week exposure, and sacrificed after 1, 2, 3, and 4 cycles of spermatogenesis (8, 16, 24, and 32 weeks, respectively). Endpoints for this study were the same as those used in the dose/time study. For the kinetics question, immediately at the end of exposure and the beginning of the recovery period, animals were housed in metabolism cages for 14 days, and urines were collected daily. Blood samples were collected retroorbitally at the end of weeks 1 and 2 to correlate with urine levels. A previous disposition study (*7*) had shown that testis boron levels were the same as blood levels.

Table 1. Selected reproductive data from F_1 Swiss mice exposed to boric acid during continuous breeding.

	Dose group			
Parameter	Control	1000 ppm	4500 ppm	9000 ppm
Litters/pair	4.71 ± 0.12 (*38*)	4.84 ± 0.09 (*19*)	2.32 ± 0.20 [a] (*19*)	– [b]
Live pups/litter	13.52 ± 0.38	13.31 ± 0.43	8.67 ± 0.76 [a]	–
Adjusted live pup weight, g [c]	1.62 ± 0.02	1.64 ± 0.02	1.39 ± 0.03 [a]	–

Data are mean ± SEM. Numbers in parentheses represent the number of pairs providing the data.
[a]Different from the control group at $p<0.05$. [b]The 9000-ppm animals had no live pups. [c]Pups weight adjusted for average litter size by least squares.

Table 2. Selected necropsy data from F_0 Swiss mice exposed to boric acid.

	Dose group			
Parameter	Control	1000 ppm	4500 ppm	9000 ppm
Terminal body weight	42.24 ± 0.80 (*39*)	42.11 ± 1.16 (*19*)	40.70 ± 0.88 (*19*)	35.69 ± 0.89 [a] (*15*)
Testis weight, mg	140 ± 3	140 ± 4	69 ± 5 [a]	20 ± 1 [a]
Rt. corpus/caput Epididymis, mg	40.65 ± 1.03	42.92 ± 0.95	32.10 ± 1.44 [a]	27.01 ± 1.82 [a]
Epididymal sperm density [b]	518.6 ± 35.8	532.4 ± 40.9	146.9 ± 26.6 [a]	2.8 ± 1.7 [a]
Epididymal sperm, % motile	78.1 ± 3.0	69.0 ± 4.5 [a]	53.3 ± 8.2 [a] (*17*)	42.9 (*1*)

Data are presented as mean ± SEM. The number of males providing the data are given in parentheses. [a]Different from the control group mean, $p < 0.05$. [b] These values are number of sperm/mg cauda × 10^3.

Results

RACB Study

Table 1 shows that boric acid effectively inhibited reproduction in mice. The high-dose group (9000 ppm) had no litters at all, while the controls averaged 4.7 litters per cohabiting pair (*4*). Mean litter size was decreased in the 4500- and 9000-ppm groups. Adjusted live pup weight was also decreased in the 4500-ppm group. These summaries hide some time-dependent changes that can be seen in the data from individual litters: the first litter in the middle-dose group (4500 ppm) was not different from controls in the number of pairs delivering, although litter size was decreased (from 12 to 9). By the third litter, fewer pairs ($n = 6$) were bearing young, and those litters were smaller than controls (ca. four pups/litter, vs ca. 14 in the control litters). Only 1 pair delivered a fourth and fifth litter in the 4500-ppm group (*5*). The 1000-ppm group was not different from controls in their fertility at any time.

When 4500-ppm-treated mice were cross-mated with controls, fertility was severely decreased in the group with the treated males; treated females bore an equal number of lighter pups, compared to control x control matings [not shown, (*4*)].

At necropsy, significant cellular changes in the male were found (Table 2). Body weight was decreased in the high-dose group, while reproductive system indices were altered in the middle- and top-dose groups. Sperm motility was decreased in all dosed groups.

There were sufficient mice to form second generation mating pairs only in the control- and low-dose groups. There was a significant 3% decrease in pup body weight when adjusted for litter size; otherwise, there were no significant effects [data not shown (*4*)].

This study clearly showed an immediate adverse effect of high-dose boric acid exposure in mice, targeting at least the male [other studies have shown developmental effects; (*8*)], and the immediate infertility in the 9000 ppm group could reflect female fertility effects as well, although this has not been fully determined. The next set of studies set out to characterize this male effect more fully.

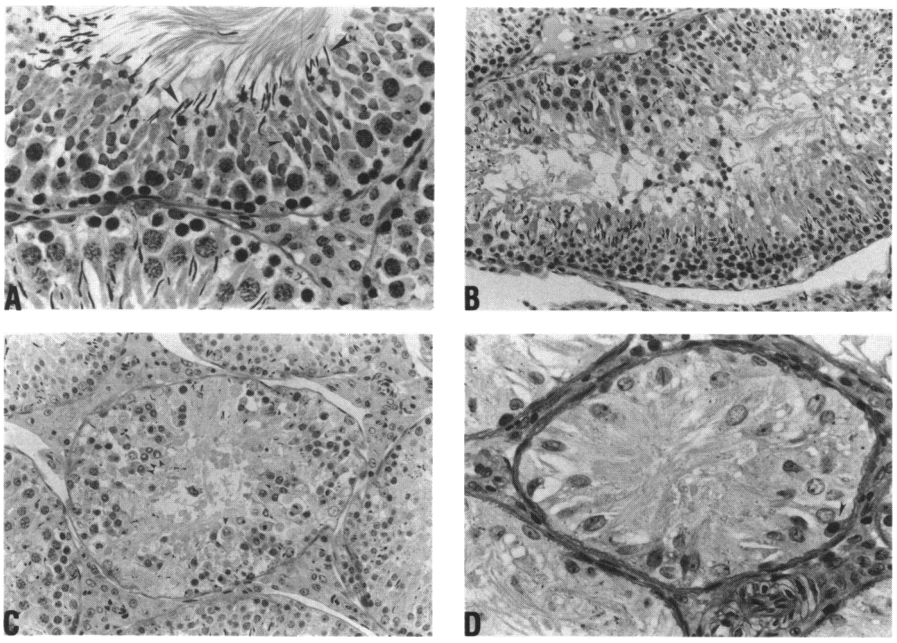

Figure 1. *A.* Section of testis from a rat treated with boric acid, showing the inhibition of spermiation (sperm release). The simultaneous presence of elongated spermatids (big arrowheads) and elogated, mature spermatids (smaller arrowheads) is aberrant. *B.* The next stage in boric acid lesion development is sloughing of germ cells. Notice the numerous round immature germ cells in the lumen of this seminiferous tubule. These also appear in sections of epididymis at this stage. *C.* Germ cell death follows epithelial sloughing. Notice the dying/dead germ cells with pyknotic nuclei and karyolysis (arrowheads), and the decreased number of germ cells within the tubule. *D.* Finally, the tubular compartment is populated only by Sertoli cells and occasional germ cell (probably a spermatogonium, arrowhead). The number of spermatogonia is the same as is thought to be in controls (*6*).

Male Pathogenesis Study

This study was performed to identify the probable target cell in the rat male reproductive system for boric acid. This study used a single concentration of boric acid in the diet (9000 ppm) and multiple sacrifice times. The first lesion appeared in some animals at day 7, and consisted of an inhibition of sperm release [Figure 1, (*6*)]. This progressed in severity, and was soon (day 21) accompanied by a disorganization of the epithelium and the release of immature germ cells. By day 28, there were some atrophic tubules that contained only residual spermatogonia and the somatic Sertoli cells. Sections of liver and kidney, examined after 28 days of dosing, contained no observable abnormalities.

Previous work has found that disruption of the androgen status of the male can disrupt spermiation (*9*). Thus, additional animals were dosed with 9000 ppm boric acid and sacrificed for testosterone analysis. A decrease in circulating testosterone levels was observed in these animals (*5*). However, several subsequent experiments failed to replicate this finding consistently (Ku and Chapin, not shown), leading us to conclude that if there is a change in testosterone levels, it is barely at the level of detectability using these methods. Indeed, others have needed more sensitive sampling techniques to define an androgen effect in male rats (*10*).

The subsequent experiments addressed questions more directed towards testicular response to toxicants and less aimed at boron-specific mechanisms. *a*) We wanted to see if we could separate the inhibited spermiation from the subsequent epithelial disorganization and atrophy. *b*) We wanted to define the lesion in terms of commonly-assessed indices of reproductive function. *c*) We wanted to see if the bone boron levels would continue to increase with exposure. *d*) We wanted see if the total dose of boric acid was more important than the dose rate in producing the lesion. *e*) We wanted to see if the testis would recover after a period of no exposure.

Dose/Time Study

This large study (*6*) can be summarized by noting the following: *a*) we found that we could, indeed, separate the inhibited spermiation (IS) from the atrophy. Animals consuming 3000 and 4500 ppm boric acid showed the IS, but did not show, during the course of the 9-week study, significant germ cell death and loss. *b*) This IS, which was a significant pathologic finding, resulted in initially normal testis weight, a slight but not significant increase in numbers of homogenization-resistant spermatids, and a significant decrease in numbers of epididymal sperm. By cellular and biochemical assays, the only "adverse effect" of this inhibited sperm release was a decrease in epididymal sperm number: organ weights and other indices were all normal. This points to the utility and necessity of informed microscopic evaluation of the testis as a tool for identifying toxicity. *c*) Bone boron rose until week 5, and plateaued proportional to dose. The bones contained 3 to 5 times the amount of boron found in blood. After week 5, there was no further increase in bone boron. *d*) Looking at the testicular lesions and comparing those to dose/time data, it became clear that dose rate was important, while total dose was not. Testis boron levels were always similar to blood boron, and for the 3000, 4500, 6000, and 9000 ppm groups, testis boron levels varied proportionately between 5.6 and 15.1 μg/g tissue. This seems reasonable for a water-soluble compound like boric acid. *e*) Finally, testes that became atrophic during dosing did not recover at all for up to 4 full spermatogenic cycles (32 weeks) after the end of dosing. This occurred even though testis and serum boron fell to background levels within 3 to 4 days of withdrawal of boric acid-containing feed. Thus, even in the absence of measurable circulating boron, no recovery was seen. Lower dose groups that did not become atrophic during exposure evidenced some recovery, but with foci of persistent atrophy. In the continually atrophic testes, spermatogonia were seen to be dividing. However, the trains of daughter cells were not able to survive, leaving the tubules with only Sertoli cells and spermatogonia.

The animals exposed to 2000 ppm in the 9-week pilot study never showed any signs of microscopic testicular damage.

Discussion

These studies show rats are more sensitive to the effects of boric acid than are mice. The calculated daily intakes of boric acid for the 9000-ppm groups of mice and rats were about 1260 mg/kg/day (mice) and 400 mg/kg/day (rats). Even taking all the other known differences between these species into account, this difference is relatively large. In terms of mg boron, this works out to about 214 and 68 mg/kg/day. These doses are relatively small compared to many other reproduc-

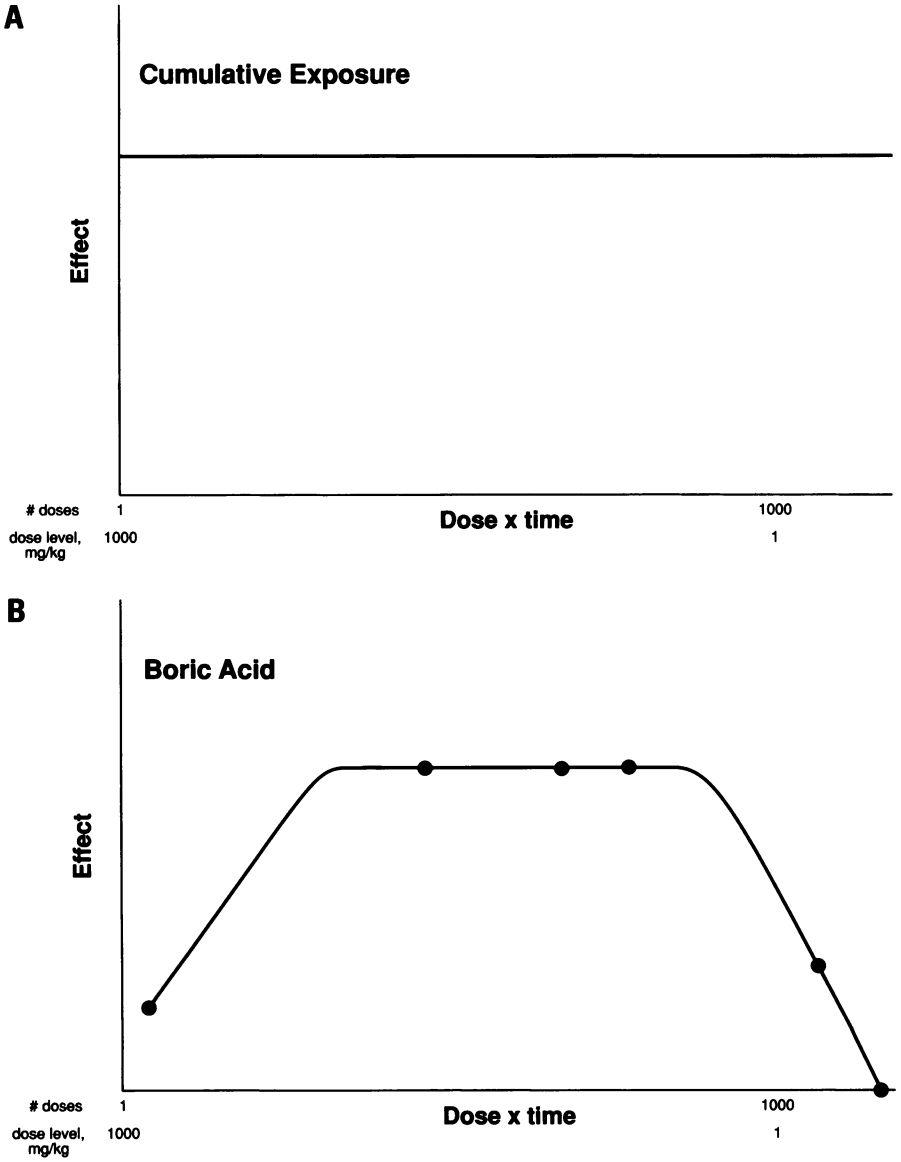

Figure 2. These curves show the effects seen when the product of dose (in mg/kg/day) × days is 1000. "Effect" in this case is inhibited spermiation, but it could be any other result of boric acid exposure. If a threshold amount of compound was important for producing an effect, then just a few doses at a very high concentration would produce the same lesion as many administrations of lower dose (top line). However, for boric acid, we can see that one very high dose produces a relatively small effect [point closest to the origin, data from Linder et al. (*11*)], then as the dose decreases but the number of doses increases, the effect rises and plateaus. Then as the dose level continues to decline (along with blood levels of B), even though the number of doses continues to increase, the effect decreases until finally there is a no effect dose level. This implies that all the dose levels that lie on the abscissa to the right are below the threshold for the effect, and will be without adverse effect on spermiation (the most sensitive effect in the rat).

tive toxicants and are within or below the effective dose range of a well-known testicular toxicant, ethylene glycol monomethyl ether (EGME) (*11*).

Overall, while boric acid appears to target the male preferentially, there are effects on the developing fetus which probably account for the lack of litters in the high-dose group in the RACB study, above (*8*). The male effects were probably responsible for the decline in fertility in the 4500 ppm group in the RACB study, as this appears to coincide temporally with developing toxicity in the testis.

The pathogenesis study was intended to identify the "target cell" in the testis. The process that was first visibly affected was spermiation, which is the interaction between Sertoli cells and elongated spermatids (the most mature testicular form of germ cells). Because this is a two-cell process, we were not able to determine the preferential target cell; electron microscopic evaluations could not identify consistent subcellular lesions in one cell type or the other. Some approaches have been pursued to further tease apart the processes involved (*12*). This lesion has been seen for some other toxicants, but no satisfactory molecular target or mechanism has been proposed or demonstrated. The lack of consistent effects on testosterone was frustrating, although others have documented some changes (*5,10*), and both these reports are of similarly small changes in testosterone levels. Our conclusion is that there may well be a depression of circulating testosterone at these elevated boron levels, but it requires serial blood samples from cannulated rats to be identified (*10*). No effect was found on isolated Leydig cells *in vitro* (*12*).

The other primary testicular lesion was germ cell death and sloughing. This is fairly common among testicular toxicants, and is a "final common pathway" for lesions that result in atrophy. Of note was the absence of Sertoli cell vacuoles or clearly dysmorphic germ cells, either of which would have allowed a clearer definition of the target cell.

A number of interesting points were made in the dose/time study. The manifestations of inhibited spermiation were subtle but logical: the gross testis indices were all normal, and only histopathology and epididymal sperm number appeared affected. It makes sense that preventing the release of sperm from the testis would decrease epididymal sperm count, but a reduction of this size had not been shown previously and in a study of this magnitude. In a report that appeared while these studies were in progress (*13*), a single administration of boric acid to rats produced a similar set of changes at one time point after dosing. The present dose/time study provides a larger picture of the interrelationship of these end points, and also documents, for the first time, that fortuitous dose selection can separate the initial parts of the lesion from those appearing later.

The disposition data indicated that boron moved with body water in soft tissues and tended to accumulate in bone. Thus, there was no accumulation of boron in testis (compared to blood). This is in contrast to an earlier claim in a paper using far fewer animals (*2*). The bone boron levels were not associated with any abnormalities in blood cell production or morphogenesis (not shown). We do not know if

these boron levels would affect the strength of the bones in the short or long term; this area remains a tantalizing possibility (*14, 15*). The rapid decrease in excreted boron just 4 days after the end of exposure, in the presence of elevated bone boron levels, suggests that bone stores do not act as significant internal depots that continue to release toxic amounts of boron in the absence of external exposure. It may be that the boron deposits in bone into two pools, one that turns over rapidly, and the other with a considerably slower turnover. In this scenario, one might expect that the high turnover rate pool would be decreased to blood levels fairly quickly (several days), while the slower turnover pool would represent a minor part of total bone boron and take much longer to exit the bone. This needs further investigation.

This study also identified that dose rate was important for the testicular lesion. Since boron levels in testis do not increase with increased exposure duration, the amount that goes into the tissue is equal to the amount that leaves; therefore, dose rate is roughly equivalent to internal concentration. If we plot data from the dose/time study together with some data from Linder et al. (*13*), we can identify an illuminating relationship (Figure 2). If the total cumulative dose were important in producing an effect (on testicular histopathology in this case, but it could be any effect: sperm production, motility, etc.), then one administration of a high dose should produce the same effect as many administrations of an equivalently lower dose (Figure 2*A*). This would be the case for compounds that are poorly excreted or bind covalently to cellular molecules. However, the curve for boric acid is quite different (Figure 2*B*). With few administrations of high doses, there is a mild effect. Lowering the dose and increasing the number of administrations (but keeping the product of dose level × duration constant) produces a significant effect (the high plateau part of the line). However, once the dose falls, even though the number of exposures is increasing, the effect diminishes until finally there is no adverse effect. This supports the concept of a threshold for boric acid. This model predicts that there would be doses that do not produce toxicity, no matter how extended the exposure duration. This relationship holds when the product of dose × days is 1000 or 2000. More experiments should be performed with the 2000, 3000, and 4500 ppm dose levels for longer times to test this hypothesis more fully. For the 9-week exposure used here, we saw no observable effects at 2000 ppm, and at least some toxicity at all the higher doses, which supports the concept of a threshold, at least within this time frame.

The importance of dose rate for lesion development has been identified for at least one other water-soluble testicular toxicant: 2,5-hexanedione (*16*).

The lack of recovery in the atrophic testes, and the persistent foci of atrophy in the "recovered" tissues, may signify either a long-term alteration in the tissue or an inherent inability of rodent testis to recover from atrophy. Rat testes have been shown to recover from numerous other chemical insults, and persistent terminal atrophy is the exception. Thus, the mechanisms that underlie persistent atrophy from a compound that is eliminated from the tissues within 72 hr are most intriguing. It may be that the direction of the investigations detailed in the following manuscript will shed some light on the molecular mechanisms of the toxicity of boric acid.

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